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14. ABSTRACT- Multiple sclerosis (MS) is an inflammatory-mediated demyelinating disease of the human central nervous system. The clinical disease course is variable and starts with reversible episodes of neurological disability (remitting–relapsing (RR-MS) stage). This transforms into a disease of continuous and irreversible neurological decline. Phosphodiesterase (PDE)-4/7 inhibitors can prevent injury-induced reductions of cAMP as well as facilitate tissue protection, anatomical repair, and functional recovery. PDE inhibitor containing nanoparticles (NP), surface modified with peptides that recognize proteins extravasated at sites of vascular disruption (clotting factors, ECM), can accumulate at regions of CNS demyelination, reducing tissue injury and promoting remyelination repair at very low drug doses. Characterization of non-functionalized polymeric (poly(ethylene glycol-b-ε-caprolactone)) NP demonstrated a size range of 28-42nm, no surface charge and a low polydispersity index. Size varied depending on amount of aminated PEG-b-PCL+peptide incorporated. NP allowed inclusion of fluorescent dyes (DiI, DiO) and the PDE-inhibitors Rolipram and BRL50481. Whereas the loading efficiency of the fluorescent dyes was high (~45% and 80% respectively), drug loading was low in comparison, but could be improved by using more polymer. The drugs were released slowly over three weeks at 37C, after which NP started to aggregate/disassemble. Functionalization of the NP with peptides (i.e. NQEQVSP, DPEAAE and NIDPNAV) improved adherence in fibrin gels (blood clots) or tissue sections of the injured spinal cord (contusion type injury, explanted 1 week after injury).

15. SUBJECT TERMS- Nanoparticles, peptides, fibrin (blood) clot, factor XIIIa, nidogen, laminin, tenascin C, versican, multiple sclerosis, drug delivery, phosphodiesterase inhibitor

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Introduction

Multiple sclerosis (MS) is an inflammatory-mediated demyelinating disease of the human central nervous system. The disease usually strikes young adults and typically begins with neurological deficits, variable periods of remission and unpredictable but clinically reversible relapses. This remitting–relapsing RR-MS stage of the disease can persist for 10–15 years and is often followed by a course of continuously progressive neurological disability referred to as secondary progressive MS SP-MS (*1*-*2*). A hallmark pathophysiological response to CNS injury or disease is a dramatic reduction in levels of the ubiquitous second messenger, cyclic adenosine monophosphate, cAMP (*3*), a critical cellular component responsible for regulating vital intracellular functions that include cell metabolism, proliferation, survival and differentiation (*4*). Levels of cAMP in cells can be reduced by a family of enzymes called phosphodiesterases (PDE) (*5*-*6*). It has been shown that the use of a PDE inhibitors prevents injury-induced reductions in cAMP after acute CNS insults (*3*, *7*) as well as facilitates significant tissue protection, anatomical repair, and functional recovery (*3*). However, despite such promise, dose-limiting side effects, primary of which are pronounced nausea and vomiting, have hampered their clinical development. Both the dose and multi-organ effects of PDE inhibitors could be reduced significantly, if the agent's release is restricted to the microenvironment of tissue pathology. To accomplish this goal a delivery system will be developed in which PDE inhibitors, encapsulated within polymeric nanoparticles, will be specifically targeted to regions of vascular disruption through surface peptide sequences on the nanoparticles that recognize extracellular matrix (ECM) proteins or clotting factors which are extravasated on damaged blood vessels (*8*-*9*).

Body

Study Objective 1: Prepare tNPs with surface-modified peptide incorporation [PD]

Task 1 (Month 1-3): *Synthesize peptides and obtain NP reagents*
1.a. *Synthesize ECM binding peptides*
The following peptides have been synthesized:

Factor XIIIa **- Fibrinogen**
Active: NH2-CGGG**NQEQVSP**-COOH
Scrambled: NH2-CGGG**VQENQPS**-COOH

Tenascin C - **Versican**
Active: NH2-CGGG**DPEAAE**-COOH
Scrambled: NH2-CGGG**PAEDEA**-COOH

Nidogen-1 – **Laminin**
Active: NH2-CGGG**NIDPNAV**-COOH
Scrambled: NH2-CGGG**IPANDNV**-COOH

NOTE: targeting peptides are taken from ECM binding sites – therefore nidogen-targeted NPs have laminin peptide ligands, Tenascin-C targeted NPs have Versican peptide ligands and FactorXIII targeted NPs have Fibrinogen peptide ligands.

PEG-b-PCL (poly(ethylene glycol-block-ε-caprolactone)) was used as the amphilic block copolymer (MW: 2000:2800, M_w/M_n: 1.15) to form the nanoparticles. N,N'-dimethylformamide (DMF) as the solvent. Peptides were conjugated to α-amino-ω-hydroxy terminated poly(ethylene glycol-block-ε-caprolactone) block copolymer (aminated PEG-b-PCL, MW: 5800:19000, M_w/M_n: 1.4) with succinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate (SMCC). Fluorescent dyes used were 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine and 3,3'-dioctadecyloxacarbocyanine perchlorate (DiI, DiO respectively).

1.b. *Formulate Nanoparticles (NPs)*
The method to prepare nanoparticles was simplified. Whereas the polymer solution had to be added drop wise to stirring PBS in the original protocol, the current protocol shortens this step to fast stirring of the two solutions. This results in a faster production of the nanoparticles. No differences in nanoparticle size between these different methods were observed.

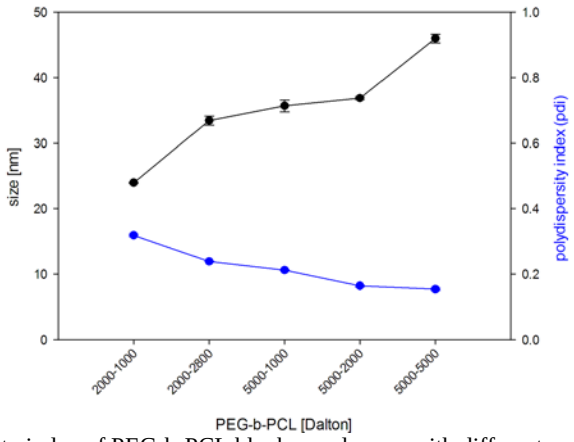

Fig.1: Size distribution and polydispersity index of PEG-b-PCL block copolymers with different molecular weight.

Dynamic light scattering was used to identify the size of the nanoparticles. We tested different sized PEG-b-PCL block copolymers with a molecular weight of 2 and 5 kDa of PEG and 1, 2, 2.8 and 5 kDa of PCL (Fig.1). Increase in polymer size lead to an increase in particle size, but a reduced polydispersity index. For further studies we choose PEG-b-PCL with a molecular weight of 2 and 2.8 kDa respectively, due to its favorable MW and nanoparticle size. Higher MW of PEG would increase the drug release (*10*) whereas the relative high amount of PCL can increase drug solubility (*11*).

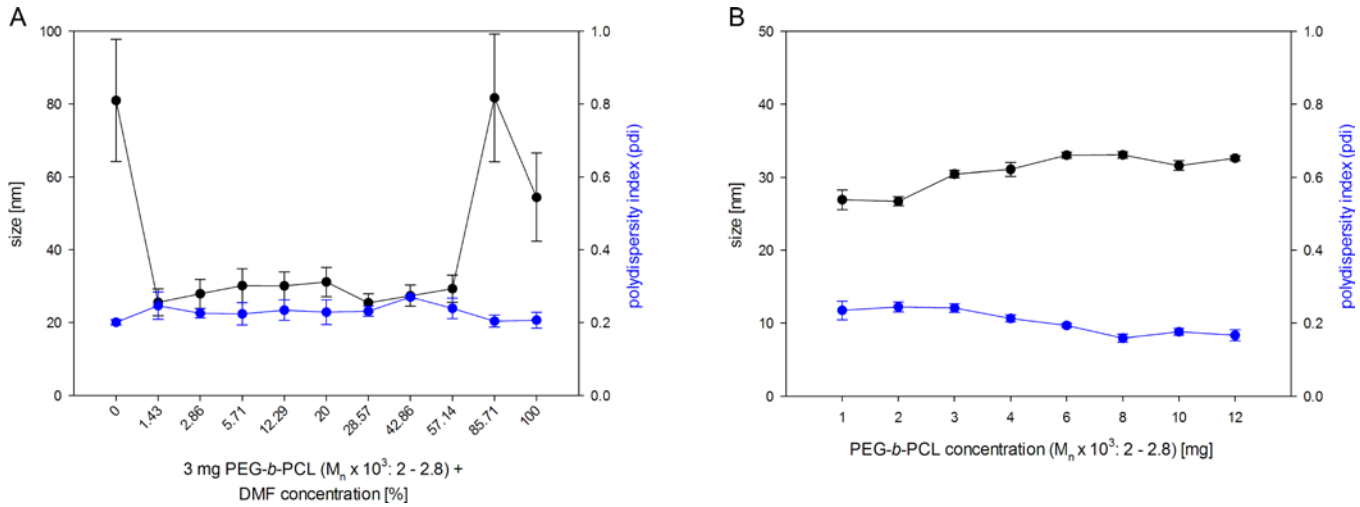

Fig.2: NP size and distribution is dependent on polymer and solvent concentration. A: Effect of DMF concentrations on nanoparticle size (n=3). B: Effect of polymer concentrations on nanoparticle size (n=3).

Low amounts of PEG-b-PCL (1-2 mg) lead to a slightly smaller nanoparticle size than higher amounts (3-12 mg), where only slight variations could be found (mean range: 30-33 nm). Furthermore, using DMF concentrations ranging from 1.43 to 57.15% resulted in similar sized NP (particle size range: 28 – 36 nm). The smallest nanoparticles were observed with 28.57% DMF, resulting in a nanoparticle size of 27.7+1.1 nm. In stark contrast using only PBS (90+47.6 nm) or high concentrations of DMF resulted in a greater nanoparticle size and high variability (85.71%: 90+58.2 nm, 100%: 67.5+50). All NP demonstrated a low polydispersity index. Polymer concentration of 3 mg and DMF concentration of 28.57% were used for further studies (Fig.2A, B).

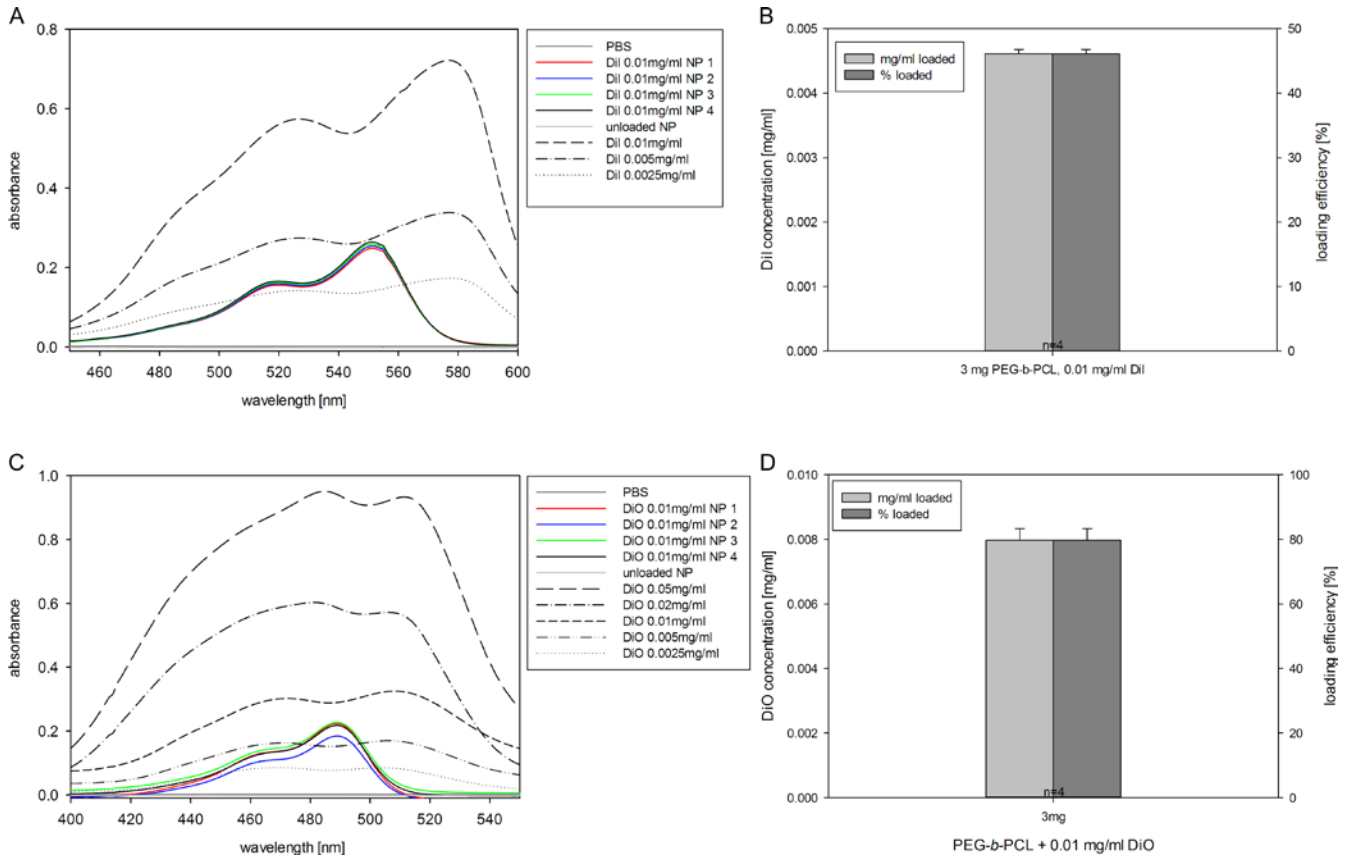

Fig.3: NP take up the fluorescent dyes DiI and DiO. A, C: Absorbance spectra for dye-laden NP, and free DiI and DiO at different concentrations. B, D: Loading efficiency and final concentration of DiI and DiO.

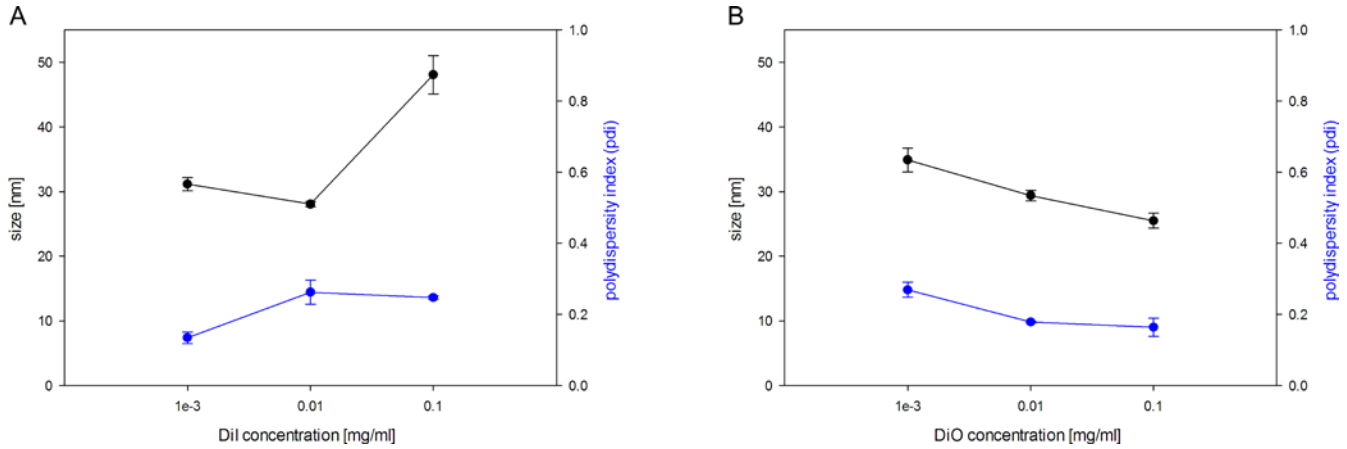

Fig.4: NP size and distribution is dependent on loading content (n=3). DiI (A) and DiO (B) NP demonstrate a low PDI with varying size, depending on loading content and concentration. Interestingly higher concentration of DiO lead to a decrease of NP size.

Both fluorescent dyes were successfully incorporated into the nanoparticles when dissolved together with the polymer in DMF as demonstrated by measuring the absorbance spectra of the dye-laden NP (Fig.3A, C). The dilution series of free dyes was used to calculate the amount taken up by the NP. The loading efficiency was good for DiI (~45%) and even higher for DiO (~80%, Fig.3B, D). Interestingly, increased DiO concentrations lead to a reduction in nanoparticle size (Fig.4B). In contrast, higher concentrations of DiI lead to an increase in mean nanoparticle size (Fig.4A).

Task 2: *Surface modify NPs with peptides*

2.a. (Month 4-9) *Incorporate different quantities of peptides into NPs and test binding efficiency*

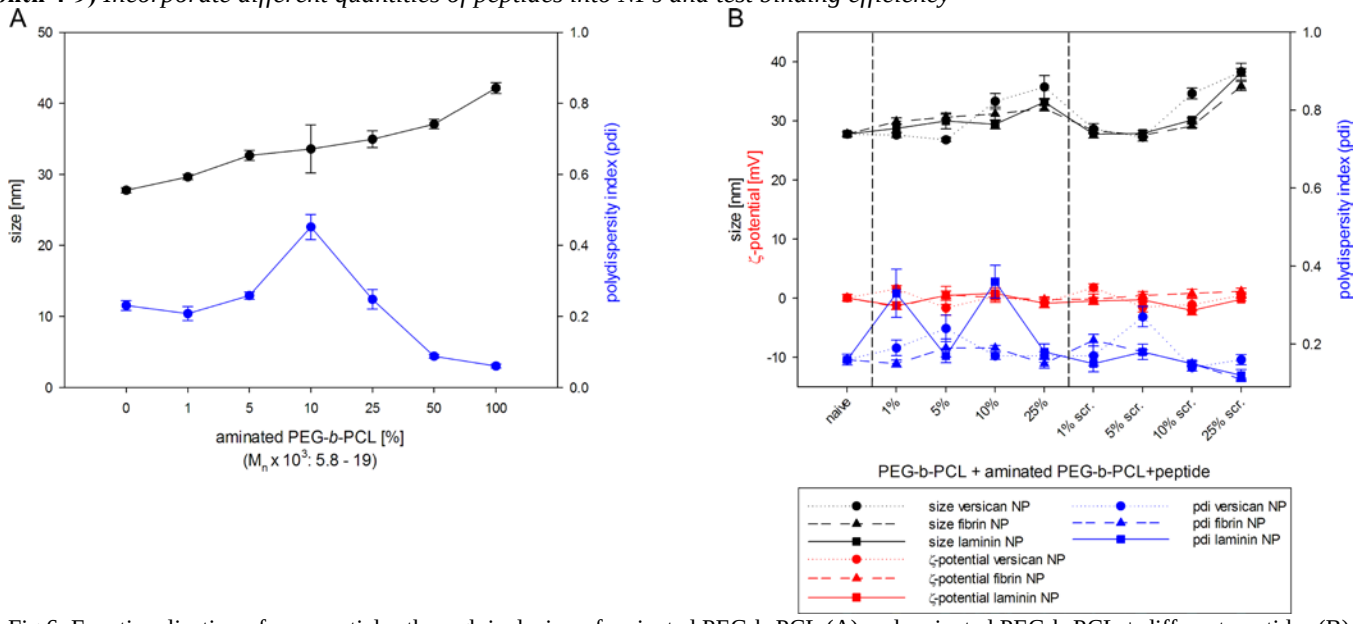

Fig.6: Functionalization of nanoparticles through inclusion of aminated PEG-b-PCL (A) and aminated PEG-b-PCL + different peptides (B) (n=3).

When the aminated PEG-b-PCL (1 to 100%) was mixed with the PEG-b-PCL containing solution before nanoparticle formation, the aminated PEG-b-PCL was successfully incorporated into the nanoparticles. Incorporation of aminated PEG-b-PCL lead to an increase in nanoparticle size, with an increase in size when higher amounts of aminated PEG-b-PCL were used (Range: ~30-42 nm, Fig.6A). The PDI initially increased, indicating a more heterogeneous size distribution of the nanoparticles, however using more than 10% aminated PEG-b-PCL lead to a reduction again (Fig.6A). Similar results were obtained with the peptide conjugated aminated PEG-b-PCL (Range: ~27-38 nm, Fig.6B). Similar to the NP, functionalized NP demonstrated no surface charge (ζ-potential) and generally a low polydispersity index (Fig.6B).

2.b. (Month 4-12) *Optimize binding and maximum peptide incorporation through NH2-PEG-PCL inclusion*

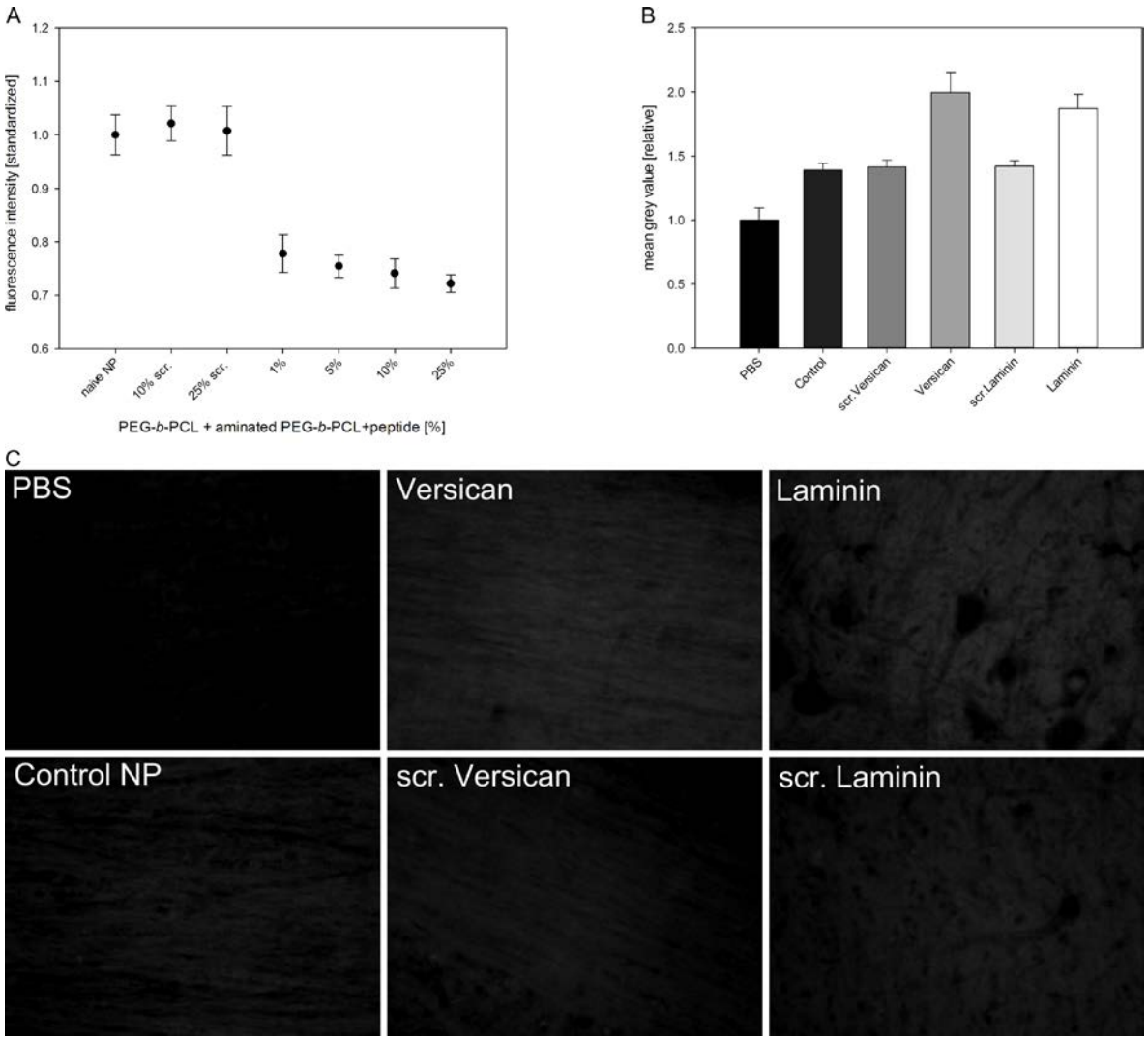

Fig.7: Peptide functionalization enhances NP binding. A: Nanoparticle release from fibrin gels *in vitro* demonstrates that fibrinogen functionalized nanoparticles bind better to fibrin gels than naive or scrambled peptide control nanoparticles (n=3). B: Laminin or Versican functionalized NP bind better to sections of the injured spinal cord (n=3). C: Fluorescence pictures of the NP bound to the spinal cord sections. Images were chosen after the average intensity value.

To test the binding capabilities of peptide functionalized NP in comparison to scrambled peptide and non-functionalized controls, NP were either embedded within fibrin gels (fibrinogen - NQEQVSP) or incubated on sections of the injured spinal cord (laminin, versican - NIDPNAV, DPEAAE). Scrambled peptide controls were used to see if the sequence itself has binding properties, even in a random order. *NP binding to fibrin gels:* The differences in size of naive and peptide functionalized NP are minimal and should not lead to different binding properties in the fibrin gel. Peptide functionalization with the factor XIIIa substrate NQEQVSP (fibrinogen) lead to a greater retention of the nanoparticles in fibrin gels. The highest retention was reached with 10% and 25% (Fig.7A). Since the best results for the fNP were obtained with 10% and 25% only those percentages of peptide incorporation were used for comparison to the scrambled peptide. The results show that the scrambled peptides are not retained within the fibrin gel and only reach values similar to the unmodified nanoparticles. Two more scrambled peptide sequences were investigated, with similar results (VENQPSQ and QVENQPS). *NP binding to spinal cord sections*: Spinal cord sections of contused spinal cords, one week after injury were used for this investigation. Peptide functionalized NP (25%, laminin and versican) demonstrated an increased binding ability to sections of the injured spinal cord compared to naive and scrambled peptide controls. Interestingly both showed unspecific binding and reached higher values than PBS (negative control, Fig.7B, C).

2.c. (Month 4-12) *Determine the protein adsorption on the NPs by incubation with fluorescent proteins.*

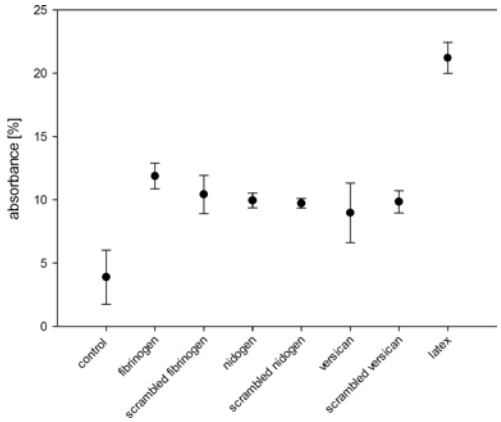

Fig.8: PEG chain prevents protein adsorption to NP. The different types of NP were incubated with bovine serum albumin (BSA) and the amount of NP that stuck to the BSA estimated by measuring the absorbance of the washed NP-BSA mix.

To determine the amount of protein adsorption on the different types of NP, they were incubated with BSA and the absorbance measured. Non-functionalized NP showed the least amount of protein adsorption (4%), due to the dense corona of PEG chains. Peptide functionalized NP were more prone to protein adsorption (9-12%), probably due to the peptide sequence (Fig.8). However naive as well as peptide-functionalized NP had a lower ability to bind proteins on its surface when compared to latex beads (21%, positive control, measured by autofluorescence).

2.d. (Month 4-12) *Organize obtained chemistry data and discuss final formulations before proceeding*
Polymer concentration of 3 mg and DMF concentration of 28.57% were determined as the optimal formulation to investigate non-functionalized NP. Inclusion of 10% and 25% aminated PEG-b-PCL+peptide demonstrated the best results regarding fibrin targeting *in vitro*. Due to the lower PDI, 25% aminated PEG-b-PCL+peptide was chosen for further studies. Similar results were obtained for the other peptides (DPEAAE, NIDPNAV), which bound to sections of the injured spinal cord. To increase the nanoparticle concentration in solution, the amount of polymer was increased to 6 mg for *in vivo* delivery.

2.e. (Month 4-12) *Prepare significant quantities of the different types of tNPs for in vivo testing [Note: tasks 2a-d will be repeated for each peptide with sequential stock production to allow for in vivo testing to take place within 4 months of project initiation]*
Different types of NP (amount and type of peptide, controls, dye) were shipped to Miami for *in vivo* testing. Stock production does not seem suitable since long term storage can affect nanoparticle, content and peptide properties. However NP can be stored for up to one month at 4°C, longer time points lead to decrease in drug content and reduce functionality of peptides. Freezing of the NP without cryoprotection leads to disassembling, freezing with a cryoprotectant to aggregation and/or surface modification.

Milestone 1: The production of characterized tNPs for each targeted protein
Non-functionalized nanoparticles and fibrinogen, laminin and versican functionalized nanoparticles are completely characterized. No peptides against myelin are available in the moment, therefore targeting of this protein is not possible with our method in the moment.

Problems encounter:

- Personnel in Brisbane arrived three month after official starting date. Furthermore to get all the necessary inductions to work in the labs delayed the onset of the practical work further.
- Long lasting defect in freeze/dryer prevented a more in depth investigations of long term storage of nanoparticles.
- No peptide sequence to bind to myelin is available momentarily, which makes targeting with our methods impossible.

Study Objective 3: Prepare tNPs with the encapsulation of PDE inhibitors or interferon-1a [PD]

Task 7: *Synthesize peptides and obtain NP reagents*
7.a. (Month 10-12) *Synthesize ECM binding peptides*
See Task 1.a.

7.b. (Month 7-12) *Formulate starPEG(sPEG) and PCL NPs*
See Task 1

7.c. (Month 13-18) *Surface modify NPs with optimal peptide(s)*
See Task 1

Task 8: *Incorporation of PDE inhibitors or interferon-1a into tNPs*
8.a. (Month 7-18) *Incorporate different quantities of drugs and test release kinetics*

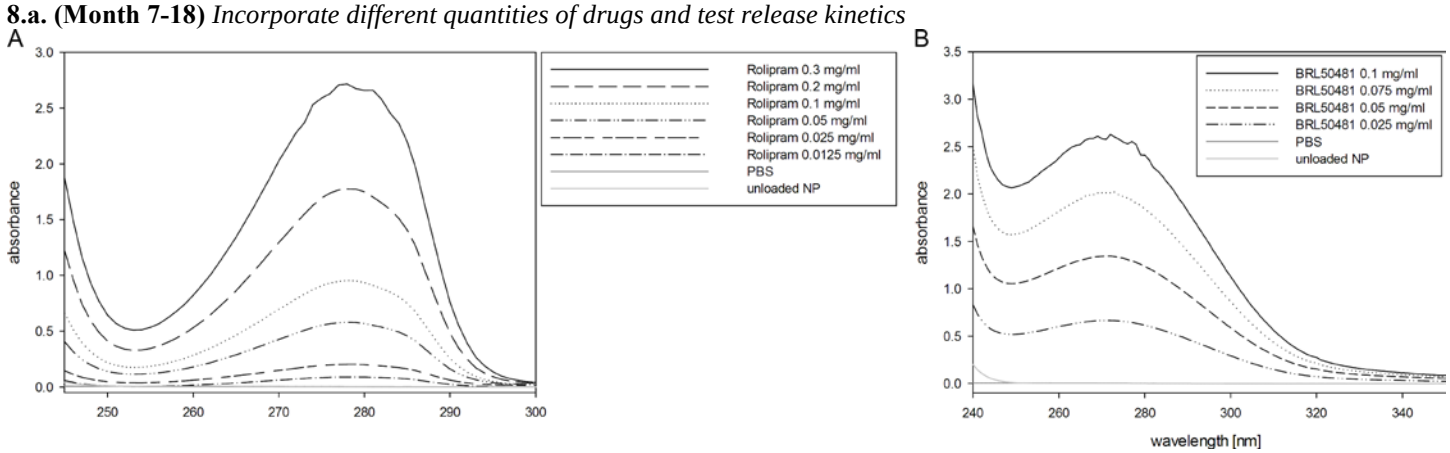

Fig.9: Absorbance spectra of Rolipram (A) and BRL50481 (B).

To measure the amount of drug taken up by the nanoparticles a dilution series of the two different PDE-inhibitors Rolipram and BRL50481 was performed and the absorbance spectra measured (Fig.9). The drug content was calculated at a wavelength of 280nm.

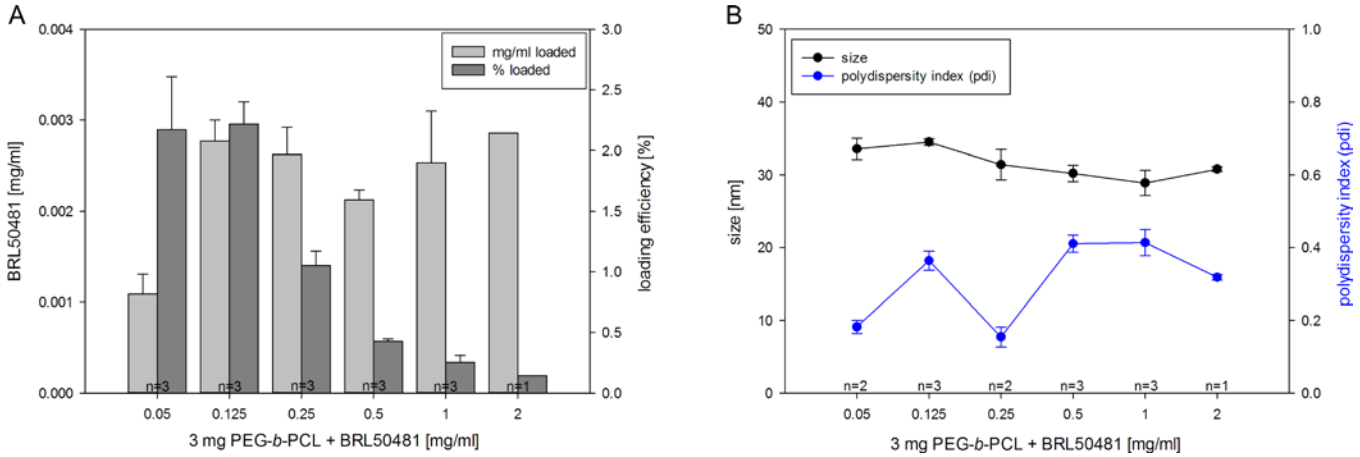

Fig.10: Loading efficiency decreases with increasing drug concentration. A: Drug loading content and efficiency of 3 mg polymer with different drug concentrations. B: NP size did not differ much with different drug concentrations, however the PDI varied, with no clear indication of a concentration effect.

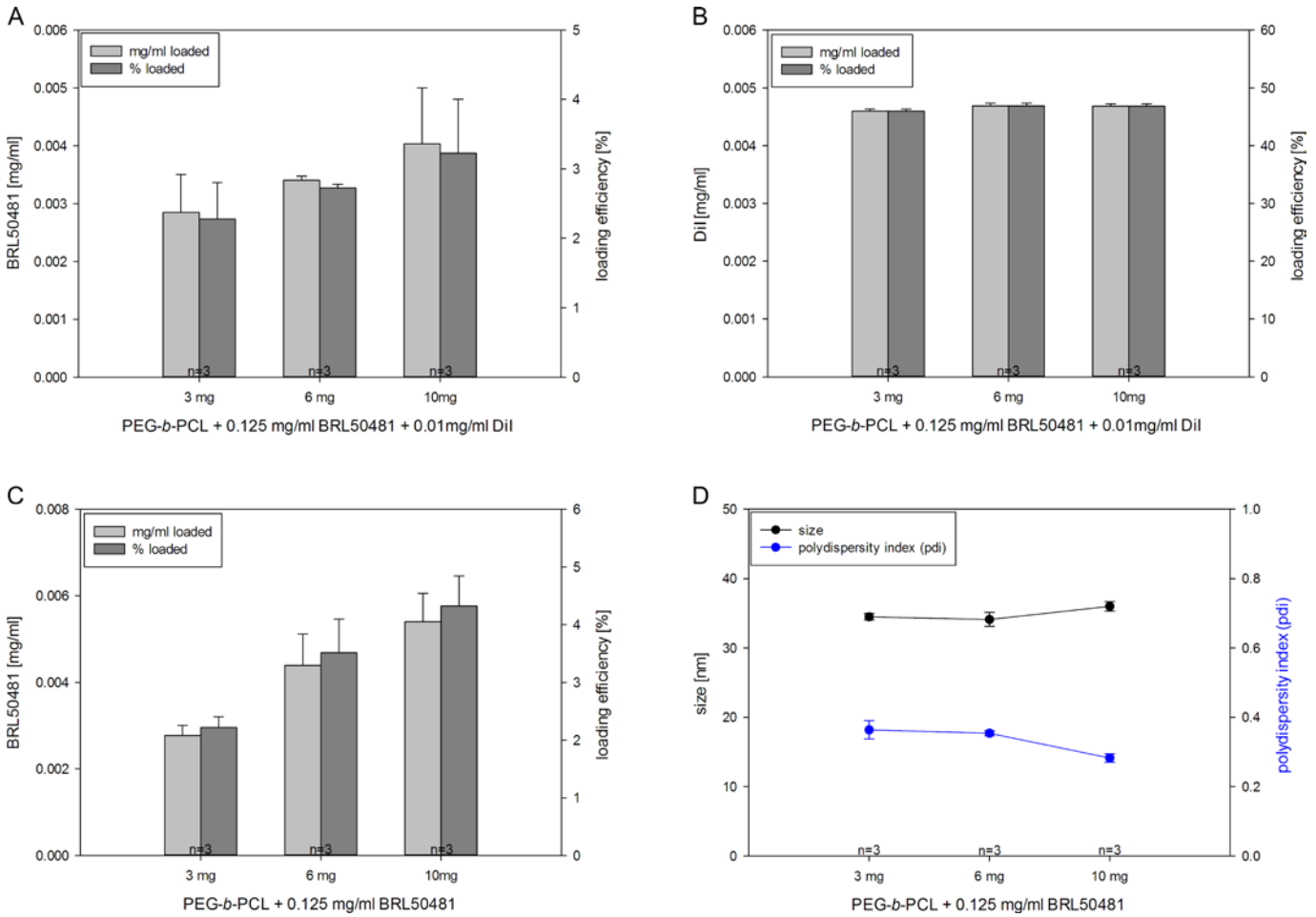

Fig.11: Fig.10: Drug (A, C) and DiI (B) loading content and efficiency of BRL50481 (0.125 mg/ml). Double loading with BRL50481 and DiI did not significantly change the amount of molecules incorporated. D: Drug laden NP size did not change when higher amounts of polymer were used.

The total amount of drug incorporated differed not much when drug concentrations above 0.05 mg/ml were used (0.021 – 0.029 mg/ml), however the loading efficiency decreased accordingly (Fig.10A). Additionally there was no effect of drug concentration on NP size (29-35 nm, Fig.10B). Some variations could be observed for the PDI (0.15-0.65), however no clear trend was noticeable (Fig.10B). Therefore further investigations were performed with lower drug amounts (0.125 mg/ml). The amount of BRL50481 could be increased, when higher concentrations of polymer were used (from 0.0028 to 0.0054 mg/ml, Fig.11A, C). No change of drug loading was observed comparing NP with or without DiI (Fig.11A, C). Similar DiI loading efficiency was comparable to non drug-laden NP (Fig11B, Fig.3B). As observed before for empty NP (Fig.2B) no change in drug-laden NP size was observed when polymer concentrations were changed (Fig.11D).

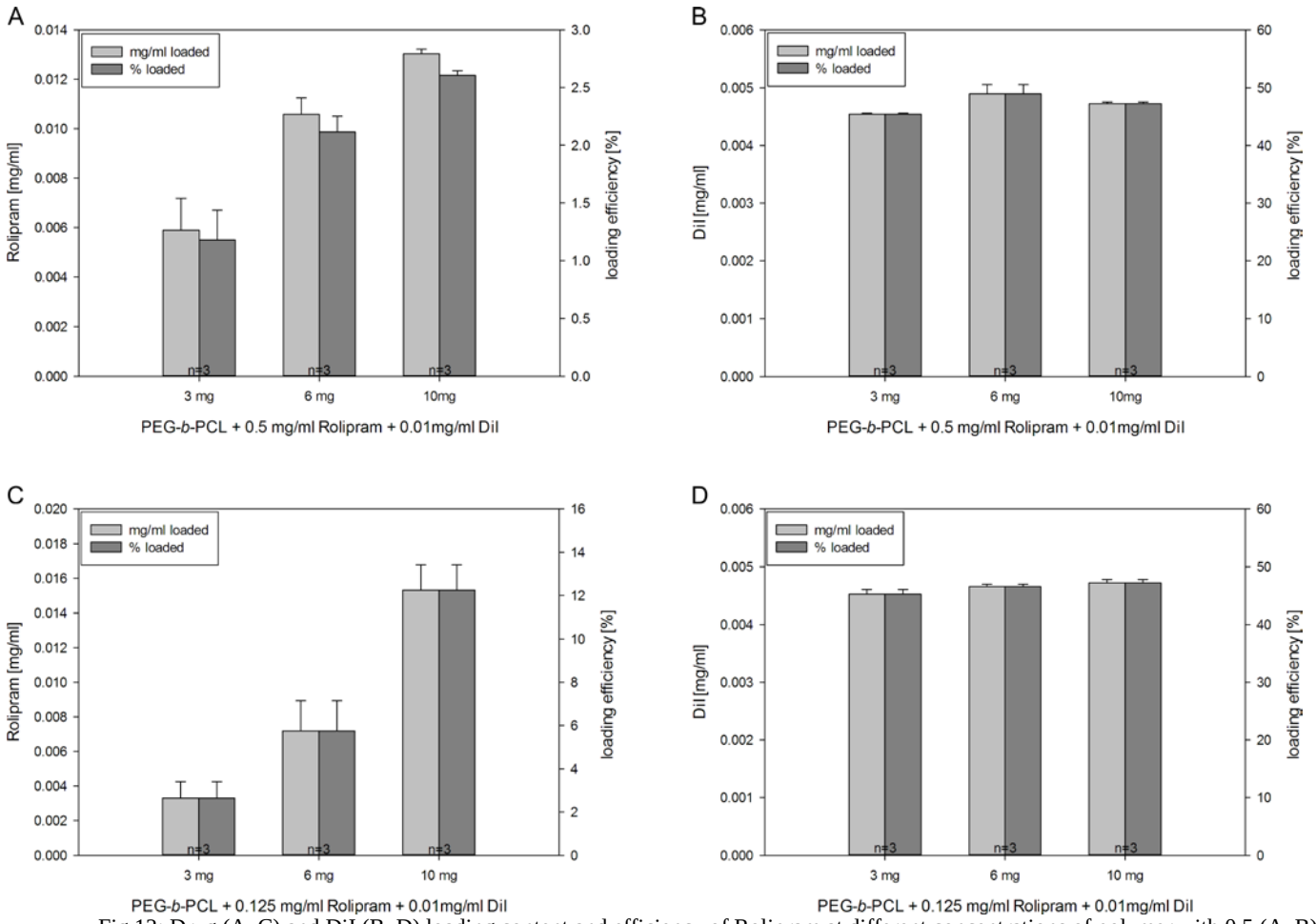

Fig.12: Drug (A, C) and DiI (B, D) loading content and efficiency of Rolipram at different concentrations of polymer with 0.5 (A, B) and 0.125 mg/ml Rolipram (C, D).

Similar to BRL50481, only low amounts of Rolipram were incorporated by the NP (Fig.10A, C). However the amount could be increased when higher concentrations of polymer were used (from 0.0033 to 0.0153 mg/ml, Fig.10A, C). Lower concentrations of Rolipram (0.125 mg/ml) resulted in similar amounts incorporated into the NP, but increased the loading efficiency (12.25 compared to 2.6%, Fig.10A, C). No change of DiI loading was observed without any (Fig.3B) or different amounts of Rolipram were used (Fig.10B, D).

8.b. (Month 10-18) *Identify the drug loading concentration that provides slow release kinetics and sustained delivery for 4 weeks in vitro*

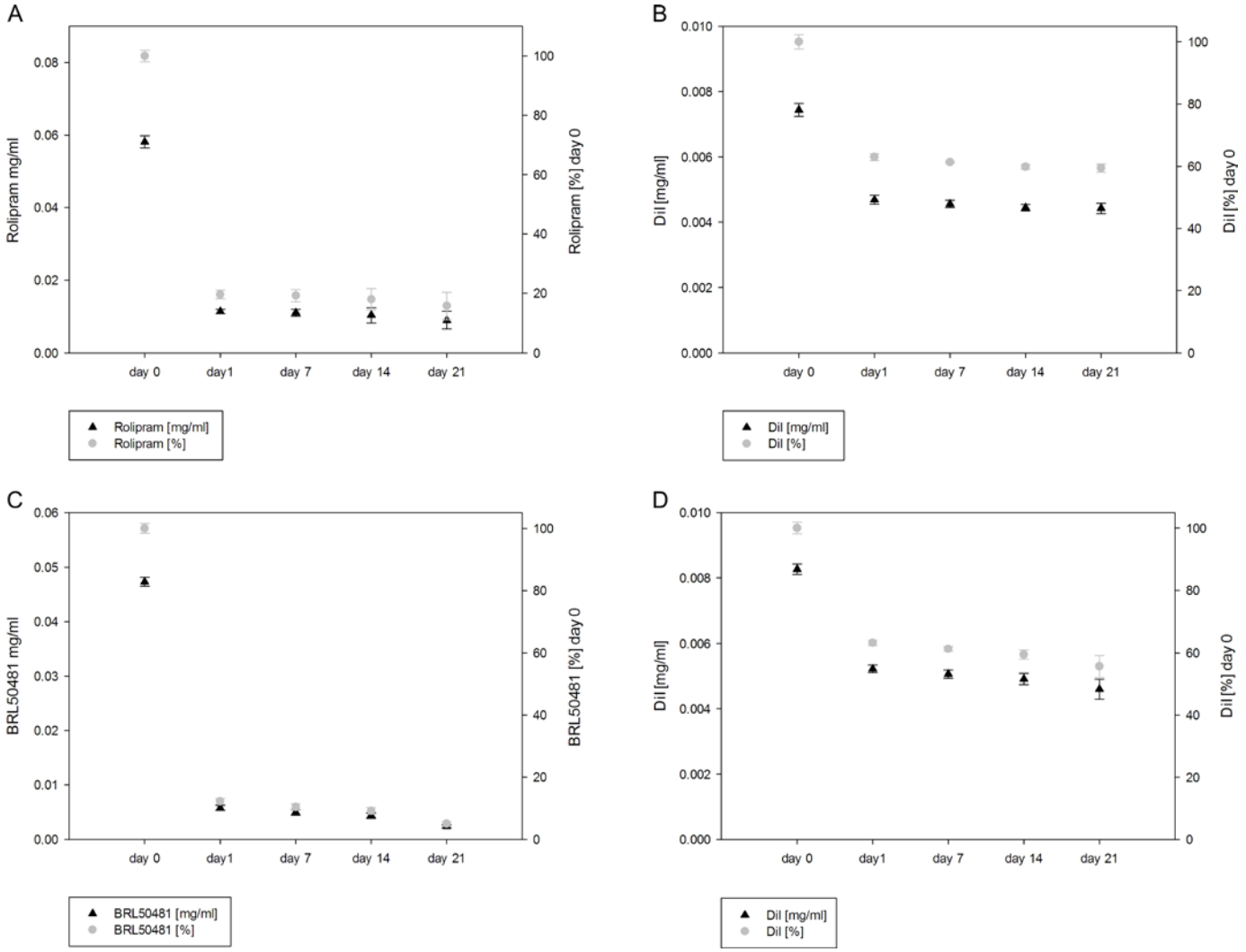

Fig.13: Drug release at 37°C. After an initial burst release, Rolipram (A, B) and BRL50481 (C, D) were slowly released over 3 weeks.

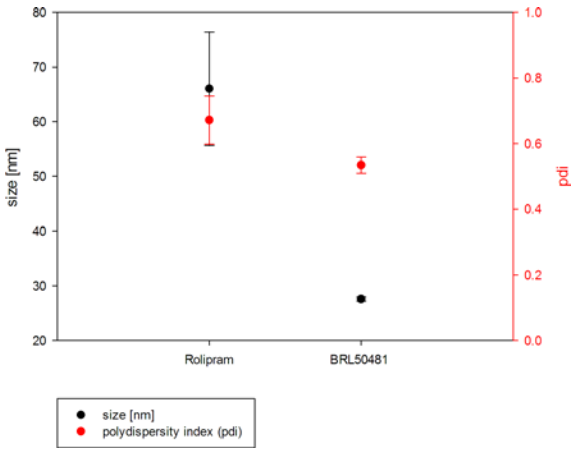

Fig.14: NP size of Rolipram- and BRL50481-laden NP, after four weeks at 37°C.

Both PDE-inhibitors as well as DiI were slowly released over a time period of three weeks, after an initial burst release (Fig.13). The burst release is probably due to surface bound molecules. DiI release was similar regardless which drug was incorporated additionally (Fig.13B, D), whereas BRL50481 was released slightly faster than Rolipram (Fig.13A, C). After four weeks nanoparticle started to aggregate, demonstrated by measuring NP size and PDI (Fig.14). Since absorbance spectra depend on NP size and the aggregation of the particle lead to a shift of the absorbance spectra in a similar fashion, analysis at later time points unfeasible (*12*). The reasons for the difference in drug (Rolipram, BRL50481) and dye (DiI, DiO) uptake and release between Rolipram and BRL50481 are not defined yet.

8.c. (Month 10-18) *Determine the protein adsorption on the NPs by incubation with fluorescent proteins.*

See Task 2c

8.d. (Month 13-18) *Organize obtained chemistry data and discuss final formulations before proceeding*
Polymer concentration of 3 mg and DMF concentration of 28.57% were determined as the optimal formulation to investigate non-functionalized NP *in vitro*. Inclusion of 10% and 25% aminated PEG-b-PCL+peptide demonstrated the best results regarding fibrin targeting *in vitro*. Due to the lower PDI, 25% aminated PEG-b-PCL+peptide was chosen for further studies. Similar results were obtained for the other peptides (laminin and versican - DPEAAE, NIDPNAV), which bound to sections of the injured spinal cord. To increase the nanoparticle concentration in solution and accompanying to increase the amount of drug available at the lesion site, the amount of polymer was increased to 6 mg for *in vivo* delivery.

8.e. (Month 13-24) *Prepare significant quantities of the different types of drug laden tNPs for in vivo testing [Note: tasks 8a-c will be repeated for each drug with sequential stock production to allow for in vivo testing to take place]*
Different types of NP were shipped to Miami for *in vivo* testing.

1. control NP with DiI (0.1 mg/ml, 0.01 mg/ml)
2. control NP with DiO (0.1 mg/ml, 0.01 mg/ml
3. control NP with Rolipram (0.125mg/ml)
4. Fibrinogen (10%, 25%, DiI)
5. Fibrinogen (10%, 25%, DiO)
6. scrambled Fibrinogen (10%, 25%, DiI)
7. scrambled Fibrinogen (10%, 25%, DiO)
8. Fibrinogen with Rolipram (25%, 0.125 mg/ml, DiI)
9. scrambled (scr) Fibrinogen with Rolipram (25%, 0.125 mg/ml, DiI)
10. Nidogen (10%, 25%, DiI)
11. scrambled Nidogen (10%, 25%, DiI)
12. Nidogen with Rolipram (25%, 0.125 mg/ml, DiI)
13. scrambled Nidogen with Rolipram (25%, 0.125 mg/ml, DiI)
14. Versican (25%)
15. scrambled Versican (25%)
16. Versican with Rolipram (25%, 0.125 mg/ml)
17. scrambled Versican with Rolipram (25%, 0.125 mg/ml)

Milestone 3: Prepared drug laden tNPs for in vivo efficacy testing after EAE
Drug-laden NP with the different types of peptides were provided.

Key research accomplishments

- Nanoparticle formation with poly(ethylene glycol-b-ε-caprolactone) block copolymer (PEG-b-PCL)
- Simplification of the nanoparticle preparation process
- Labelling of nanoparticles with fluorescent dyes
- Incorporation of the PDE-inhibitors Rolipram and BRL50481 within the nanoparticles
- Aminated (PEG-b-PCL) integration into (PEG-b-PCL) nanoparticles
- Peptide functionalization of aminated nanoparticles
- Improved fibrin gel retention with fibrinogen functionalized nanoparticles, compared to standard PEG-b-PCL nanoparticles
- Improved binding to sections of the injured spinal cord of laminin and versican functionalized nanoparticles
- Characterization of the different types of nanoparticles (size, PDI, ζ-potential, binding, protein adsorption)

Reportable outcomes

Oral presentation at the World Biomaterials congress 2012.

Manuscript in Preparation. **Peptide Functionalized PEG-block-PCL Nanoparticles for Targeting Fibrin Clots. To be submitted to Journal of Controlled Release.**

Conclusion

Nanoparticles could be successfully generated from poly(ethylene glycol-b-ε-caprolactone) block copolymer. NP had a size range of 28-36 nm when polymer or solvent concentrations were changed. Optimal nanoparticle formation in regard to size distribution was determined as 28.57% DMF and 3 mg polymer concentrations (6mg were used for *in vivo* investigations). NP allowed inclusion of fluorescent dyes (DiI, DiO), drugs (Rolipram, BRL50481) and aminated PEG-b-PCL. The peptides (i.e. NQEQVSP, DPEAAE and NIDPNAV) could be conjugated to the aminated PEG-b-PCL and integrated into the nanoparticles. Peptide (i.e. NQEQVSP, DPEAAE, NIDPNAV and their scrambled controls) functionalized NP (fNP) were slightly larger in size then non-functionalized NP (range 30-42 nm). All NP had no surface charge and a low polydispersity index.
Fibrinogen (NQEQVSP) fNP showed a better adherence then non-functionalized NP to fibrin gels, with 10% and 25% functionalization demonstrating the best adherence. Laminin (NIDPNAV) and Versican (DPEAAE) functionalized nanoparticle (25%) demonstrated better adherence to sections of the injured spinal cord (contusion type injury, explanted 1 week after injury).
Drug concentration was low, but could be increased with increasing amount of polymer. Variation of initial drug concentration had no effect on drug incorporation. The NP released the drug slowly over a time period of up to 4 weeks. Drug release was measured for up to three weeks, at four weeks aggregation of the particles made it difficult to determine the drug content. However even at four week some NP for further release were detectable.

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Appendices- none